Passive Transfer of Serum Antibodies Induced by BBG2Na, a Subunit Vaccine, in the Elderly Protects SCID Mouse Lungs Against Respiratory Syncytial Virus Challenge

Hélène Plotnicky-Gilquin,* Dominique Cyblat-Chanal,* Liliane Goetsch,* Christine Lacheney,* Christine Libon,* Thierry Champion,* Alain Beck,* Hélène Pasche,† Thien N. Nguyen,* Jean-Yves Bonnefoy,* Nancy Bouveret-le-Cam,* and Nathalie Corvaïa*

*Centre d’Immunologie Pierre Fabre, 5 Av. Napoléon III, F74 164, Saint-Julien-en-Genevois, France; and †Institut de Recherche Pierre Fabre, F31 319, Jean Rostand, Labège INNOPOLE Cédex, France

Respiratory syncytial virus (RSV) is the single most important cause of serious lower respiratory tract disease in infancy and early childhood, responsible for bronchiolitis, hospitalizations, and more than 2500 deaths in 1997 in the United States (Shay et al., 1999, 2000). It also represents a major risk factor for the incidence of asthma and sensitization to common allergens (Sigurs et al., 1995). Despite the induction of cellular and Ab responses, a protective immunity is not achieved after the first encounter of the virus and reinfections are frequent throughout life (Hall et al., 1991; Henderson et al., 1979).

This virus is actually recognized as a determinant factor for pneumonia and exacerbation of chronic pulmonary or cardiac diseases in adults (Dowell et al., 1996; Sommerville, 1963). In addition, adults of advanced age may be at particular risk for RSV-related illness. In the United States, approximately 15–60,000 hospitalizations and 2–7000 deaths due to RSV-associated pneumonia occur annually among the elderly (Han et al., 1999). The cost of RSV-related hospitalizations in this population is estimated between $150 and $680 million per year. Thus, the use of an RSV vaccine in the elderly and subjects with underlying conditions would be largely cost-effective (Gessner, 2000).

To date, no RSV vaccine is available. We described a new approach to develop such a vaccine (Power et al., 1997). An RSV-A (Long strain) G protein-derived fragment (BBG2Na) was expressed as a part of a fusion protein in Escherichia coli (Murby et al., 1995). This recombinant molecule appeared to be a promising vaccine candidate for several reasons. It contains a carrier protein, BB (Libon et al., 1999), fused to residues 130–230 of RSV G protein (G2Na), that includes a stretch of residues (164–173) that are completely conserved in all known RSV isolates (Collins et al., 1996). BBG2Na is highly immunogenic in rodents and induces sterilizing lung protection against both RSV-A and RSV-B infections (Power et al., 1997). Immunopathologic responses, such as those observed in the 1960s during clinical evaluation of a formalin-inactivated whole RSV preparation (Fi-RSV), were never observed in murine priming models of enhanced pathology (Corvaïa et al., 1997; Plotnicky-Gilquin et al., 1999a). In addition, BBG2Na remained highly immunogenic in newborn mice despite the presence of RSV-specific maternally-acquired Abs (Siegrist et al., 1999), as well as in RSV-primed mice (Goetsch et al., 2000).

The safety and the efficacy of BBG2Na in different animal models, including primates (Trudel et al., 1998), prompted the molecule to clinical studies. Phases I and II were carried out (Power et al., 2001; LeCam et al., 2000) and a Phase III in the elderly population is currently
going on. Adjuphos-precipitated BBG2Na was well tolerated and immunogenic both in young adults (Power et al., 2001) and in subjects aged over 60 years (LeCam et al., 2000). Anti-G2Na-specific Ab responses were multiplied by a twofold factor or more in up to 68% of the subjects. The twofold seroconversion rate against RSV-A after 1 injection of 100 or 200 μg of BBG2Na was up to 22% of the subjects.

The aim of our present study was to test whether BBG2Na administration in the elderly during the Phase II clinical trial induced anti-RSV-A protective Abs. For this purpose, the protective efficacy of pre- and postimmunization sera of 11 subjects immunized with BBG2Na or with placebo were evaluated by transfer at serial dilutions into SCID mice before challenge with RSV-A. The Ab profiles were also determined by pepscan analysis.

RESULTS

Characterization of the antibody responses

Three hundred eight subjects, aged 60–80 years, were enrolled in the clinical trial and assigned to vaccine or placebo groups in a randomized, double-blind, and controlled fashion (LeCam et al., 2000). Eleven of them were selected on the basis of the Ab responses detected in the sera before the first immunization and 28 days thereafter. The selected subjects were injected once with 100 or 200 μg of BBG2Na or the placebo. The prevaccination anti-G2Na IgG titers were quite similar except for the subject PER\textsuperscript{+}; they comprised between 67 and 137 arbitrary units (AU) ml (mean of SEL serum titers: 91 ± 19 AU/ml) (Table 1). Four weeks after the first immunization, the subjects who received BBG2Na showed a 7- to 37-fold increase in their anti-BBG2Na Ab titers (mean of V serum titers: 7258 ± 3458 AU/ml versus mean of SEL serum titers: 521 ± 318 AU/ml). This increase was attributable to the induction of IgG to BB, the carrier protein, and G2Na. For all subjects, anti-G2Na IgG titers were multiplied at least by a fourfold factor. Similarly, BBG2Na-immunized subjects also showed an increase in their serum of anti-RSV A IgG titers, although the magnitude of this increase did not necessarily correlate with that observed for the anti-G2Na IgG titers. RSV-A neutralization titers remained relatively stable except for individual POI\textsuperscript{+} and PER\textsuperscript{+} for whom the VN titers increased more than six and five times, respectively. This increase was attributable to the induction of IgG to BB, the carrier protein, and G2Na. For all subjects, anti-G2Na IgG titers were multiplied at least by a fourfold factor.

Reactivities of sera with synthetic peptides

To analyze the IgG responses and determine epitope usage before and after immunization with BBG2Na in the
selected subjects, SEL and V sera were screened in pepscan using a series of overlapping dodecapeptides that spanned G2Na. Results of two representative subjects, one placebo and one immunized, sharing similar anti-G2Na Ab profiles before immunization, are shown in Figs. 1A–1D. BIE received the placebo, while GAU was immunized with BBG2Na. SEL sera of the two patients shared several zone reactivities (Figs. 1A and 1C): the major ones were located between aa 158 and 169 or 160 and 171, while minor reactivities were also observed with 12-mer peptides spanning aa sequences 141 to 159, 177 to 198, 204 to 215, 209 to 220, and 213 to 224.

The reactivities observed after immunization with placebo were comparable with those observed before immunization, with the exception of a new peak on peptide 163–174 (Fig. 1B). The common reactivities were all detected at comparable intensity before and after immunization, consistent with the similarity in the anti-G2Na Ab titers measured in SEL and V sera of this subject (Table 1). In contrast, although the major zone reactivities were also conserved between SEL and V sera of the BBG2Na-immunized subject, after immunization, these zones were detected at a higher intensity, and enriched with several additional peaks: peptides 130–141, 137–148, 139–150, 161–172, 164–175, 173–184, 176–187, and 180–191 (Fig. 1D). However, these peaks were not specific for BBG2Na, as they were also observed in some preimmunization sera (not shown), indicating that they could be induced after repeated natural RSV infections. Some of these reactivities (underlined in Fig. 1D) coincided with previously described protective epitopes in mice (Plotnicky-Gilquin et al., 1999). Thus, immunization with BBG2Na boosted the anti-RSV Ab responses against multiple epitopes located on the G protein.

**Protective efficacy of SEL and V sera after transfer into SCID mice**

To compare the protective efficacy of pre- and postimmunization sera of BBG2Na- and placebo-injected individuals, we set up a passive transfer model in SCID mice. To do so, we initially used sera of young adult

---

**FIG. 1.** The reactivities of pre- (SEL) and postimmunization (V) sera from individuals BIE (immunized with placebo) and GAU (immunized with BBG2Na) against overlapping 12-mer peptides spanning the region containing residues 130–230 of RSV-A G protein were evaluated by pepscan analysis at dilution 1/5000. Reactivity peaks are numbered to facilitate direct comparison of the various sera, the numbers corresponding to the first amino acid residue of the implicated peptide. The level of the background values is indicated by a line. Peptides coinciding with previously described protective epitopes in mice are underlined.
healthy volunteers of the Phase I study. Our previous studies demonstrated that RSV-A protection can be accomplished in mice, following passive Ab transfer using either the intraperitoneal (i.p.) or the intranasal (i.n.) route (Power et al., 1997; Plotnickiy-Gilquin et al., 1999b). Accordingly, i.p. and i.n. transfer of serially diluted sera from Phase I trial into SCID mice resulted in reduction of lung RSV-A titers in a dose-dependent manner (not shown). In both cases, a sterilizing protection could be achieved with the undiluted human sera comparing favorably with the control murine anti-RSV-A serum. In contrast, when these sera were diluted 1/100, they had no more effect as lung RSV-A titers of mice were similar to those of mice treated with irrelevant Abs (i.e., the murine anti-BB serum). However, to obtain the same level of protection, the i.n. transfer (performed with 50 μl/mouse) required significantly less serum than the i.p. transfer (performed with 200 μl/mouse). Hence, we chose to test the sera of the 11 selected individuals of Phase II using the i.n. route.

As shown in Fig. 2A, although the maximum level of lung infection (given by the group of mice treated with the anti-BB serum) varied with the experiment (from 3.8 ± 0.3 to 5.2 log_{10} TCID_{50}/g lung), all SEL and V sera induced a dose-dependent reduction of lung RSV-A titers after i.n. instillation into SCID mice. In addition, at a dilution of 1/10, the efficacy of SEL sera varied considerably with the subjects. However, the postimmunization sera of all BBG2Na-immunized subjects significantly reduced lung RSV-A titers compared with the corresponding preimmunization sera, at least at one of the three dilutions tested (P < 0.05). In contrast, at either dilution, there was no significant difference in lung RSV-A titers between mice treated with the pre- and postimmunization sera of the subjects vaccinated with placebo (Fig. 2B). Similar results were obtained in separate experiments, as shown by lung RSV-A titers measured in the first and in the second test performed with SEL and V sera of subject LAI.

To investigate the influence of the route of serum transfer on the protection results, the protective efficacy of the pre- and postimmunization sera of subjects GAU (both vaccinated with BBG2Na) and CAL (immunized with placebo) were also evaluated after transfer by i.p. injection. As shown in Table 2, at a dilution of 1/2, after immunization, the sera of GAU reduced lung RSV-A titers in mice more efficiently than the corresponding preimmunization sera (P < 0.05). In contrast, lung RSV-A titers were similar in mice treated with SEL and V sera of BIE, the placebo-immunized individual.

In conclusion, our results demonstrated that the anti-RSV-A protective efficacy of the serum was significantly increased in the elderly after immunization with BBG2Na, and not with the placebo, in comparison with the sera obtained from these subjects before immunization. This effect was demonstrated by passive transfer of the pre-and postimmunization sera into SCID mice, using either the i.p. or the i.n. route.

**DISCUSSION**

RSV is increasingly recognized as an important pathogen in the elderly, with a rate of associated disease probably close to that of influenza (Dowell et al., 1996; Falsey et al., 1995). Immunoprophylaxis and immunotherapy with hyperimmune globulins or with humanized monoclonal Abs against RSV are very expensive and their use is restricted to high-risk newborns or infants. Antiviral therapy is controversial and of limited effectiveness (Kahn, 2000; Zambon, 1999). Therefore, developing an RSV vaccine for the elderly appears to be the solution of choice to control excess morbidity and mortality due to RSV in this population. In the present article, we demonstrated that BBG2Na, an RSV G protein-derived recombinant vaccine, successfully induced protective Abs against RSV infection in the serum of individuals aged over 60 years, as shown in a passive immunization mouse model.

The in vivo anti-RSV-A protective efficacy of human sera was previously investigated in the 1980s, in a cotton rat model (Prince et al., 1985). In this model, sera of RSV-convalescent individuals and purified Abs were transferred by i.p. injection into rats before RSV challenge, at the dose of 0.5 ml/10 g of body weight. This represents at least 4 to 5 ml of serum required for the transfer into a single animal. Since only small volumes (generally ≤ 1 ml) of SEL and V sera of each individual were available in our hands, we set up a transfer model using SCID mice instead of the cotton rats. After challenge with RSV, these immunoincapable mice develop a chronic infection in lungs (Plotnickiy-Gilquin, personal observation). In addition, the transfer of human sera might be facilitated by their lack of Abs. Passive immunizations were performed either by the i.p. or by the i.n. route, the latter condition allowing us to make the comparisons with extremely low final volumes of sera (i.e., <50 μl for the i.n. versus at least 1800 μl for the i.p. route). Under either condition, pre- and postimmunization sera of placebo-treated individuals showed similar protective activities. In contrast, postimmunization sera of BBG2Na-immunized subjects displayed a significantly increased protective efficacy against RSV-A lung infection, after transfer into SCID mice, compared with the corresponding preimmunization sera.

Surprisingly, no correlation could be clearly established between the IgG responses, the neutralization titers, and the effectiveness of the different sera. This might be due at least in part to the relative variability observed in the level of RSV-A infection of our mouse model. However, it also probably reflects the complexity of individual responses raised against the different RSV proteins, after repeated natural infections with different virus strains, these responses being more or less modified upon immunization with BBG2Na.

The lack of correlation also suggests that increasing
the neutralization titer is not a prerequisite to improve the anti-RSV protective efficacy of human sera. This hypothesis is supported by the observation that several anti-G2Na monoclonal Abs that failed to neutralize RSV infection \textit{in vitro} were nonetheless able to induce lung sterilizing protection \textit{in vivo} after transfer into SCID mice.
TABLE 2
Protective Efficacy of Sera after i.p. Transfer into SCID Mice

<table>
<thead>
<tr>
<th>Serum transfer</th>
<th>GAU⁺</th>
<th>CAL⁺</th>
<th>BIE⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEL undiluted*</td>
<td>1.48 ± 0.04⁺</td>
<td>2.12 ± 0.90⁺</td>
<td>2.2 ± 0⁺</td>
</tr>
<tr>
<td>SEL 1/2</td>
<td>4.1 ± 0.28</td>
<td>3.4 ± 0.34</td>
<td>3.95 ± 0.25⁺</td>
</tr>
<tr>
<td>SEL 1/5</td>
<td>4.45 ± 0.14⁺</td>
<td>3.5 ± 0.37</td>
<td>4.45 ± 0.38⁺</td>
</tr>
<tr>
<td>V undiluted</td>
<td>1.48 ± 0.04</td>
<td>1.8 ± 0.24</td>
<td>2.2 ± 0.25⁺</td>
</tr>
<tr>
<td>V 1/2</td>
<td>3.41 ± 0.23⁺</td>
<td>2.6 ± 0.30⁺</td>
<td>3.8 ± 0.28⁺</td>
</tr>
<tr>
<td>V 1/5</td>
<td>4.33 ± 0.19⁺</td>
<td>3 ± 0.40</td>
<td>4.45 ± 0.25⁺</td>
</tr>
<tr>
<td>anti-BB</td>
<td>4.6 ± 0.20⁺</td>
<td>4.05 ± 0.30⁻</td>
<td>4.85 ± 0.14⁻</td>
</tr>
<tr>
<td>anti-RSV-A</td>
<td>1.8 ± 0.70⁺</td>
<td>1.45 ± 0.0⁻</td>
<td>1.5 ± 0.12⁻</td>
</tr>
</tbody>
</table>

* Preimmunization (SEL) and postimmunization (V) sera were transferred pure or diluted 1/2 or 1/5 in PBS before i.p. injection of 200 µl into SCID mice. The control anti-BB and anti-RSV-A sera were transferred at anti-BB and anti-RSV-A ELISA titers of 5 log10.

⁺ Mean RSV-A titers ± SD expressed in log10 (TCID50)/lung and calculated from groups of five to six mice.
⁻ P < 0.05. Comparisons were made between groups treated with SEL and V sera of the same subject, used at the same dilution.

(Materials and Methods)

**Virus, cells, and ELISA antigens**

Respiratory syncytial virus subgroup A (RSV-A) (Long strain, ATCC VR-26, American Type Culture Collection, Rockville, MD) propagation in HEP-2 cells (ECACC 86030501, European Collection of Animal Cell Cultures, Porton Down, Salisbury, U.K.), as well as the production of viral protein and uninfected cell ELISA antigens, were performed as previously described (Power et al., 1997). Viruses were harvested after 48–72 h by scraping attached cells into the medium, centrifuging the suspension at 460 g for 15 min, and collecting the supernatant as the virus stock. RSV was stored at −196°C until use.

**Immunization procedure and sera**

BBG2Na was purified (>99%) from *E. coli* cell lysates. The vaccine was supplied in two separate vials including BBG2Na freeze-dried with mannitol and aluminum phosphate (ADJU-PHOS, Superfos, Denmark) suspended in water. The formulation was reconstituted just before use and injected into the deltoid muscle. Vaccinees received one or two doses of 50, 100, or 200 µg of BBG2Na (patient surnames are noted “⁺⁺”), each containing 560 µg aluminum, or of placebo (saline) (patient surnames are noted “⁺”), at a 28-day interval. Blood samples were harvested before the first injection (designed “SEL” sera) and 4 weeks after the first injection (designed “V” sera). These sera were kept at −80°C until use. The sera of two nonimmunized adult healthy volunteers were also used to set up the passive transfer experiments.

**Immunological assays**

**ELISA.** Serum total IgG levels were determined using RSV-A (Long) or B (B1 wt; ATCC VR 1400) infected HEP-2 cell lysates, BBG2Na, G2Na, or BB as coating antigens at

(Immunological assays)
previously optimized protein concentration (Power et al., 2001). Serial serum dilutions were incubated with the coating antigens. Bound IgGs were detected with HRP-conjugated anti-human IgG (Biosource International, Camarillo, CA). Total IgG levels in test sera against viral antigens were expressed as arbitrary units per ml (AU/ml) based on standard curves generated with an internal working standard (a pool of sera of BBG2Na-immunized volunteers from Phase I trial) following absorbance reading at OD450.

Virus neutralization (VN) assay. Virus neutralization assay was performed as previously described (Power et al., 2001). Briefly, serial twofold dilutions of heat-inactivated (56°C × 30 min) test serum were prepared in triplicate in DMEM with 1% fetal bovine serum (FBS) (BioWhittaker, Walkersville, MD) in 96-well flat-bottomed plates (Greiner) (50 μl/well). Approximately 100 tissue-culture infectious dose 50 (TCID50) RSV-A or B in 50 μl were added per well and plates were incubated at 37°C for 1 h. Following incubation, 10^4 and 5 × 10^3 HEp-2 cells were added per well for RSV-A and B VN assays, respectively. After 6–8 days at 37°C, cytopathic effect was scored for each well. Fifty percent VN titers were calculated by the Reed and Muench method (1938). Two reference human sera (average VN titers 1/40) were treated in a similar fashion and were used as the basis to calculate arbitrary VN AU titers/ml for each test serum sample, using the following formula: (VN titer of test serum/average VN titer of two reference sera) × 100.

Animals

Female SCID BALB/c mice, aged 7–8 weeks, were purchased from IFFA CREDO (L’Arbresle, France) and kept under specific pathogen-free conditions. They were fed rat and mouse maintenance diet A04 (UAR, Villeminois-sur-Orge, France) and water ad libitum and were housed and manipulated according to French and European guidelines.

Protection studies

For passive transfer experiments, mice were anesthetized by intramuscular (i.m.) injection of 2.5 ml/kg of a 4/1 mixture (v/v) of ketamine (Imalgène 500) (Rhône Mérieux, Lyon, France) and xylazine (Rompun at 2%) (Bayer, Puteaux, France). Pre- and postimmunization sera were transferred pure or diluted in PBS, by i.p. injection of 200 μl, or by i.n. instillation of 50 μl per mouse, using 5–6 mice per dilution. Control mice for infection and for protection were treated, respectively, with an anti-BB or an anti-RSV-serum, diluted at an anti-BB or an anti-RSV-A ELISA titer of 5 log10. These murine polyclonal antisera were produced by immunizing BALB/c mice three times at 2-week intervals by i.p. injections of 20 μg BB in 20% aluminum phosphate or by i.n. instillation of 10^5 TCID50 RSV-A. Three weeks after the last immunization, the mice were sacrificed and exsanguinated by cardiac puncture. Blood samples were collected in serum separation tubes (Beckton–Dickinson, Meylan, France) and centrifuged at 1850 g for 10 min, and the sera were pooled. The titers of the resulting anti-BB and anti-RSV-A antisera were determined as previously described (Power et al., 1997). These sera were stored at −20°C until use in passive transfer experiments.

Eighteen hours after the serum transfer, the animals were challenged with 10^3 TCID50 RSV-A i.n., as described above. The mice were sacrificed 5 days later, after anesthesia and exsanguination by cardiac puncture. Lung removal, lung homogenate preparation, and virus titration were undertaken as previously described (Power et al., 1997). The limit of detection for lung tissues was ≤1.45 log10 TCID50/g of lung, except where insufficient lung homogenate was available. When no virus was detected, actual detection limits were used for statistical analyses. Thus, standard deviations < 0 were occasionally recorded for lung titers of some virus-free animal groups. Organs were considered protected when virus titers were reduced by at least 2 log10 relative to anti-BB-treated control mice.

Pepsan analysis

Ninety overlapping 12-mer peptides spanning residues 130–230 (G2Na) of the human RSV-A G protein were synthesized on nonclivable derivatized rods (Mimotopes, Clayton Victoria, Australia) according to established procedures. The peptides were tested for their reactivities with sera by ELISA. Briefly, nonspecific binding was blocked by incubation for 1 h at 37°C with phosphate-buffered saline (PBS) containing 0.1% Tween (Sigma) and 1% gelatin. The rods were subsequently incubated at 4°C overnight with the sera, washed three times, and incubated 1 h at room temperature (RT) with a HRP-conjugated goat anti-human antibody (1/5000) (Southern Biotechnology Associates, Birmingham, AL). After washing four times, the blocks of pins were placed in a 96-well microtiter plate (Nunc, Roskilde, Denmark) containing 100 μl tetramethyl benzidine (Dynatech, Chantilly, VA). The reaction was terminated 7 min later with 100 μl/well of 1 M H2SO4. Optical densities (OD) were measured at 450 nm. A reactivity was analyzed as a positive signal when the measured optical density was higher than twice the background value; the background value was determined as the mean of the 10 minor optical densities measured in the plate.

Statistics

Statistical analyses were done using the t test or Kolmogorov–Smirnov of the Statigraphic software program (Manugistics, Rockville, MD), depending on the
normal distribution of the values. Probability values greater than 0.05 were considered insignificant.

ACKNOWLEDGMENTS

We thank Francis Derouet, Valéry Moine, and Lydie Reyv for technical help and we are grateful to Dr. Andrés McAllister for critically reviewing the manuscript.

REFERENCES


